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# Preserving organelle vitality: peroxisomal quality control mechanisms in yeast

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## Abstract

Cellular proteins and organelles such as peroxisomes are under continuous quality control. Upon synthesis in the cytosol, peroxisomal proteins are kept in an import-competent state by chaperones or specific proteins with an analogous function to prevent degradation by the ubiquitin–proteasome system. During protein translocation into the organelle, the peroxisomal targeting signal receptors (Pex5, Pex20) are also continuously undergoing quality control to enable efficient functioning of the translocon (RADAR pathway). Even upon maturation of peroxisomes, matrix enzymes and peroxisomal membranes remain subjected to quality control. As a result of their oxidative metabolism, peroxisomes are producers of reactive oxygen species (ROS), which may damage proteins and lipids. To counteract ROS-induced damage, yeast peroxisomes contain two important antioxidant enzymes: catalase and an organelle-specific peroxiredoxin. Additionally, a Lon-type protease has recently been identified in the peroxisomal matrix, which is capable of degrading nonfunctional proteins. Finally, cellular housekeeping processes keep track of the functioning of peroxisomes so that dysfunctional organelles can be quickly removed via selective autophagy (pexophagy). This review provides an overview of the major processes involved in quality control of yeast peroxisomes.

## Introduction

The eukaryotic cell is distinguished from prokaryotes (bacteria, archaea) by the separation of a wide variety of processes in specific compartments, termed organelles. An important class of organelles are the peroxisomes, which play vital metabolic functions in all eukaryotes. By definition, these organelles harbour hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>)-producing oxidases together with catalase (CAT), an enzyme that decomposes this highly reactive oxygen compound. Another generalized function of peroxisomes is  $\beta$ -oxidation of fatty acids, which is conserved from yeast to humans. However, peroxisomes may harbour highly diverse metabolic pathways. In fungi, these range from those that are catabolic in nature and allow the use of various unusual carbon and nitrogen sources for growth (e.g. methanol, alkanes, purines, D-amino acids, cf. Table 1) to biosynthetic pathways such as lysine biosynthesis in *Saccharomyces cerevisiae* and penicillin production in the filamentous

fungus *Penicillium chrysogenum* (for a recent review, see Schrader & Fahimi, 2008 and the references therein).

During the last two decades, our knowledge of the biology of peroxisomes has strongly increased, especially from studies in yeast species. The molecular mechanisms of organelle formation and proliferation as well as their autophagic removal have been unraveled at the molecular level. Peroxisomal quality control systems and the role of dysfunctional organelles in cell viability/cell death are relatively novel topics in peroxisome research. Recent data suggest that dysfunctional peroxisomes may contribute to ageing (Terlecky *et al.*, 2006). Ageing can be defined as the deterioration of cells in time, accompanied by a loss of viability. Ageing cells progressively accumulate damaged or nonfunctional macromolecules (e.g. proteins, lipids) as a result of enhanced rates of the formation of these molecules coupled to insufficient removal or repair processes (cellular housekeeping) (Bergamini *et al.*, 2004; Gilca *et al.*, 2007). Reactive oxygen species (ROS) are key players in causing

damage to macromolecules in aerobic living cells. In eukaryotes, mitochondria are usually seen as the major producers of ROS, formed as a byproduct of oxidative phosphorylation. However, peroxisomes may also considerably contribute to ROS formation, because these organelles generate  $H_2O_2$  as a byproduct of oxidative carbon/nitrogen metabolism. Therefore, in addition to the well-accepted role of mitochondria in cell viability and ageing, peroxisomes may contribute significantly to such processes as well.

**Table 1.** Peroxisomal enzymes that generate ROS

Enzyme	Substrate	ROS
(1) Acyl-CoA oxidases	Fatty acids	$H_2O_2$
(2) Urate oxidase	Uric acid	$H_2O_2$
(3) Xanthine oxidase	Xanthine	$H_2O_2$ , $O_2^{\cdot-}$
(4) D-amino acid oxidase	D-proline, D-alanine, D-aspartate, N-methyl-D-aspartate	$H_2O_2$
(5) Pipecolic acid oxidase	L-Pipecolic acid	$H_2O_2$
(6) Sarcosine oxidase	Sarcosine, pipecolate	$H_2O_2$
(7) L-Alpha-hydroxy acid oxidase	Glycolate, lactate	$H_2O_2$
(8) Polyamine oxidase	N-Acetylspermine/spermidine	$H_2O_2$
(9) Sulfite oxidase	Sulfite	$H_2O_2$
(10) Alcohol oxidase	Methanol	$H_2O_2$

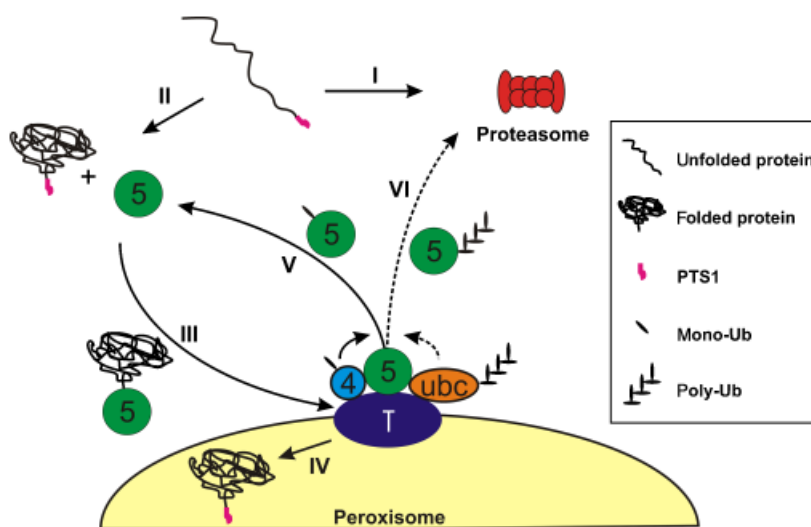
Oxidases (1)–(8) have been identified in peroxisomes of both lower and higher eukaryotes, including fungi. The last two enzymes are confined to either plant peroxisomes (9) or fungal peroxisomes (10).

In this contribution, we present an overview of the current knowledge on how the cell prevents the formation of dysfunctional peroxisomes. We mainly focus on housekeeping processes related to proteins, which are relevant in case of peroxisomes, and discuss the housekeeping processes that are involved in removing nonfunctional damaged peroxisomal components or entire organelles.

Because of constraints of space, only in a few cases will we address the quality control mechanisms that occur in other organelles such as mitochondria, endoplasmic reticulum (ER) and nucleus (for reviews, see Von Mikecz, 2006; Malhotra & Kaufman, 2007; Neutzner *et al.*, 2007; Anelli & Sitia, 2008; Friguet *et al.*, 2008; Nakatsukasa & Brodsky, 2008; Starkov, 2008).

## Quality control of peroxisomal proteins in the cytosol before import

In the cytosol, newly synthesized peroxisomal proteins undergo continuous quality control to prevent accumulation of misfolded or inactive proteins (Fig. 1; Martinez-Vicente *et al.*, 2005). For newly synthesized proteins, in most cases, molecular chaperones are important to facilitate proper folding into functional structures. In the absence of these chaperones, proteins can misfold, which may ultimately lead to the formation of toxic protein aggregates in the cell. The importance of chaperones is corroborated by their increased expression under stress conditions.



**Fig. 1.** Schematic representation of Pex5 recycling and quality control during peroxisomal matrix protein import in yeast. Peroxisomal proteins are synthesized on free polysomes in the cytosol, where quality control processes ensure that the proteins either become degraded by the UPS (I) or are properly folded (II). During peroxisomal import, the PTS1 receptor Pex5 (5) binds newly synthesized, folded PTS1 proteins in the cytosol and assists in their entry into the organelle via the peroxisomal translocon (T; III). Upon release from the receptor, the PTS1 protein enters the peroxisomal matrix (IV). Under physiologically optimal conditions, monoubiquitination of Pex5 by the E2 enzyme Pex4 (4) drives efficient recycling of Pex5 molecules to the cytosol to enable another round of import (V). However, under specific conditions, Pex5 may remain stuck at the export site. To free the translocon from interfering Pex5 molecules, these become polyubiquitinated by Ubc4 (ubc) and transported to the proteasome for turnover (VI).

So far, very little is known on the role of cytosolic chaperone proteins in peroxisome biogenesis and peroxisomal protein import. Hettema *et al.* (1998) showed that in *S. cerevisiae*, the cytosolic DnaJ-like protein Djpl is important for peroxisomal matrix protein import. In mammalian cells, a cytosolic Hsp70 protein was shown to be required for association of an artificial substrate with peroxisomes (Walton *et al.*, 1994). In a later study, it was demonstrated that import of microinjected peroxisomal proteins can result in coimport of chaperone molecules of the hsc70 family (Brocard *et al.*, 2003). The exact molecular function of these chaperones has remained elusive so far. They most likely are not important to keep proteins unfolded, as peroxisomal proteins can be imported in a folded state.

Cytosolic quality control of peroxisomal proteins is most likely performed by the ubiquitin–proteasome system (UPS), one of the major proteolytic machineries of eukaryotic cells (cf. Fig. 1). This multicatalytic protease complex recognizes proteins that are modified by several ubiquitin molecules covalently bound to the substrate protein (polyubiquitination; reviewed in Ciechanover, 2005). Because the UPS only resides in the cytosol and the nucleus, proteins localized to other cell compartments are generally not assumed to serve as its substrates. A well-characterized exception is the ER, where the endoplasmic reticulum-associated protein degradation (ERAD) system designates misfolded luminal and membrane proteins as targets for ubiquitination, resulting in their removal from the ER and subsequent degradation by the proteasome (Nakatsukasa & Brodsky, 2008). Recent reports propose the existence of systems analogous to ERAD associated with other organelles such as mitochondria (the outer mitochondrial membrane-associated protein degradation pathway; OM-MAD; reviewed in Neutzner *et al.*, 2007) and peroxisomes [quality control of import receptors; RADAR (receptor accumulation and degradation in the absence of recycling); Fig. 1; Léon & Subramani, 2007]. However, most organelles including peroxisomes harbour specific proteases, which may recognize and degrade damaged/exhausted proteins inside the organelle, instead of a UPS-related system.

### Quality control during peroxisomal matrix protein import

A set of unique proteins, the so-called peroxins (encoded by *PEX* genes; Distel *et al.*, 1996), is involved in peroxisome formation. Of the 32 known *PEX* genes, > 20 play a role in formation of the peroxisomal membrane or in matrix protein import, whereas the others are involved in the regulation of peroxisome abundance, size and distribution (reviewed by Kiel *et al.*, 2006).

### Quality control of peroxisomal membrane proteins (PMPs)

PMPs are synthesized in the cytosol and post-translationally inserted into the peroxisomal membrane (reviewed by Fujiki *et al.*, 2006). However, recent data suggest that some PMPs may first travel to the ER and subsequently sort to peroxisomes, a process that may involve vesicle transport processes (cf. Karnik & Trelease, 2007). It is now widely accepted that Pex3 and Pex19 are pivotal molecules in the post-translational import of PMPs (Fujiki *et al.*, 2006). Pex19 has been suggested to have a chaperone function for PMPs in many studies. PMPs translated *in vitro* in the presence of purified Pex19 remain soluble, whereas in the absence of this peroxin, they form aggregates. Additionally, *S. cerevisiae* *pex19* mutants have strongly reduced PMP levels, most likely resulting from degradation of poorly folded protein molecules by the UPS (Hettema *et al.*, 2000). Thus, Pex19 binding increases the half-life of newly synthesized PMPs and maintains their membrane insertion-competent conformation. Probably, Pex19 binding stabilizes the newly synthesized PMPs by shielding their hydrophobic membrane-spanning domains. It is likely that other cytosolic chaperones are also involved in the quality control of PMPs in the cytosol.

Additionally, once inserted into the peroxisomal membrane, PMPs might be proteolytically removed again. In the methylotrophic yeast *Hansenula polymorpha*, the focal levels of the PMP Pex14 are reduced significantly during the late phases of growth, possibly as a result of removal by the UPS (de Vries *et al.*, 2006). Furthermore, in *H. polymorpha*, the PMP Pex3 is specifically degraded before removal of the entire organelle from the cytoplasm by macropexophagy (Bellu *et al.*, 2002). Most likely, this process is mediated by the UPS.

### Peroxisomal matrix protein import

Like PMPs, peroxisomal matrix proteins are synthesized on free ribosomes in the cytosol and are imported post-translationally. A remarkable feature of peroxisomal matrix protein import is that the translocon has the capability to accommodate folded or oligomeric proteins, suggesting the presence of a large – possibly transient – pore. Indeed, it was demonstrated that many peroxisomal matrix proteins fold and oligomerize before import into peroxisomes (see Titorenko *et al.*, 2002 and the references therein). Consequently, a significant portion of the protein quality control processes for peroxisomal matrix proteins are thought to take place before import of the proteins into the organelle. This notion is consistent with the observation that Hsp60- or Hsp70-type chaperones are apparently absent inside yeast and mammalian peroxisomes. So far, only plant peroxisomes

have been demonstrated to harbour chaperones (Wimmer *et al.*, 1997; Ma *et al.*, 2006).

In specific cases, proteins other than the classical chaperones (such as heat shock proteins) may keep specific newly synthesized peroxisomal proteins in an import-competent state (cf. Titorenko *et al.*, 1998). An extensively studied example of this is the protein pyruvate carboxylase (Pyc1) in methylotrophic yeast species (Ozimek *et al.*, 2003). In these organisms, the octameric peroxisomal flavoenzyme alcohol oxidase (AO) is the first enzyme in the utilization of methanol as the sole source for carbon and energy ( $\text{CH}_3\text{-OH} + \text{O}_2 \rightarrow \text{CH}_2\text{O} + \text{H}_2\text{O}_2$ ). Unlike many other oligomeric peroxisomal proteins, AO is imported into peroxisomes in a monomeric form (Stewart *et al.*, 2001), which contains the cofactor FAD. Once imported, spontaneous octamerization of monomers occurs to form the active AO enzyme. Pyc1 is thought to be important for binding FAD to AO monomers and possibly also to prevent assembly into octamers in the cytosol. This is important because AO octamers cannot be imported into peroxisomes (Faber *et al.*, 2002). Mutants lacking Pyc1 in both *H. polymorpha* and *Pichia pastoris* are unable to grow on methanol, have strongly reduced AO activities and accumulate FAD-lacking AO monomers in the cytosol (Ozimek *et al.*, 2003). Remarkably, it is not the enzyme activity of the Pyc1 enzyme – which replenishes the tricarboxylic acid (TCA) cycle with oxaloacetate – but rather the protein itself that has this chaperone-like function (Ozimek *et al.*, 2003).

Two receptor proteins are known that each recognize one of the two yet known peroxisomal targeting signals (PTSs): the PTS1 receptor Pex5 and the PTS2 receptor complex Pex7/Pex20 (see Kiel *et al.*, 2006). Conceptually, peroxisomal protein import can be divided into four steps (for a recent and detailed review, see Platta & Erdmann, 2007). First, the soluble PTS receptor binds its cargo protein in the cytosol and guides it to the peroxisomal membrane. Second, the receptor–cargo complex associates at a peroxisomal docking complex, which consists of three peroxins: the PMPs Pex13, Pex14 and Pex17. The third step involves the actual translocation of the cargo across the peroxisomal membrane through a yet unknown protein translocon. This step also includes cargo–receptor dissociation, which is thought to be mediated by Pex8. Finally, the receptor is recycled to the cytosol for further rounds of import, a step that includes a quality control mechanism. Translocation and receptor recycling require the function of at least 10 membrane-bound peroxins (see Kiel *et al.*, 2006).

The actual mechanism of peroxisomal protein translocation is still under debate. The observation that both PTS receptors have a dual location in the cytosol and inside peroxisomes led to the speculation that protein import takes place via a so-called extended shuttle mechanism in which the receptor/cargo complex is fully translocated across the

membrane, followed by cargo/receptor dissociation and subsequent export of the receptor back to the cytosol (van der Klei & Veenhuis, 1996). Indeed, data have been presented demonstrating that the receptor molecules actually are in direct contact with the lumen of the organelle (Dammai & Subramani, 2001). More recently, Erdmann & Schliebs (2005) proposed that the Pex5–cargo complex might directly insert into the peroxisomal membrane where Pex5 molecules are arranged to form the actual peroxisomal protein translocation pore. In this scheme, the other peroxisomal membrane-borne peroxins are thought to be mainly required for receptor recycling, rather than for the actual import process. This model was supported by the finding that a considerable portion of membrane-associated Pex5 behaves as an intrinsic membrane protein even when unable to bind the proteins of the putative docking complex (Kerssen *et al.*, 2006).

### Quality control of import receptors during peroxisomal protein import

The import receptors of peroxisomal matrix proteins can be degraded by the UPS, a pathway that has been designated RADAR (Léon *et al.*, 2006) and has been observed in *S. cerevisiae*, *H. polymorpha* and *P. pastoris* (Platta *et al.*, 2004; Kiel *et al.*, 2005a, b; Léon *et al.*, 2006). RADAR is involved in degradation of two related proteins that function in the sorting of PTS1 proteins (Pex5) and PTS2 proteins (Pex20, the coreceptor of Pex7) (cf. Kiel *et al.*, 2006). The pathway appears to be mainly active when proper recycling of these receptor molecules is inhibited and they remain present at the peroxisomal export site, thus possibly obstructing the import machinery (Fig. 1).

Recently, Erdmann's group (Platta *et al.*, 2007) was able to separate the RADAR pathway from the regular Pex5 recycling pathway. They proposed that following PTS1 import, membrane-bound Pex5 can be modified either by poly- or by monoubiquitination. During normal receptor recycling, a conserved cysteine residue in the N-terminus of Pex5 becomes monoubiquitinated (mUb), a process that is mediated by the E2 enzyme Pex4 (Williams *et al.*, 2007). Subsequently, Pex5–mUb molecules are recognized by the AAA peroxin Pex1 that enables release of the receptor from the peroxisomal membrane into the cytosol. However, so far, there is no evidence that Pex1 can actually recognize the mUb moiety. The RADAR pathway becomes activated when export of Pex5 from peroxisomes is obstructed (e.g. in yeast *pex1*, *pex4* and *pex6* mutants; Kiel *et al.*, 2005a, b). Under these conditions, Pex5 becomes polyubiquitinated (pUb) at one or more lysine residues near its N-terminus, a process dependent on the cytosolic E2 enzyme Ubc4. Pex5–pUb molecules are then pulled out of the membrane by as yet unidentified components and directed to the proteasome

for degradation. A process similar to that described here for Pex5 is believed to occur for Pex20, the coreceptor of Pex7 (Léon *et al.*, 2006). In mammals, the E2 enzyme Pex4 is absent and members of the E2D/UbcH5 family of E2 enzymes are involved in monoubiquitination of the Pex5 receptor during its recycling (Grou *et al.*, 2008). Moreover, the RADAR system seems to be conserved in mammals as well, because Pex5 levels are drastically reduced in cell lines that are blocked in normal receptor cycling [e.g. in CG-1 (*pex1*) or CG-4 (*pex6*) fibroblasts; Dodt & Gould, 2001]. Thus, the cell ensures the proper functioning of peroxisomal matrix protein import via RADAR, which can be considered as a part of cellular housekeeping related to peroxisomes.

### Threats of peroxisomal metabolism: ROS formation and repair of ROS-related damage

Recent data indicate that peroxisomes are important factors in ROS production (Bener Aksam *et al.*, 2008). In yeast, peroxisome deficiency (Jungwirth *et al.*, 2008) was shown to result in necrotic cell death. Also, dysfunctional organelles may lead to imbalances in ROS production and degradation. The accumulating effects of the damages will, as for mitochondria, result in cell death. Also, internal signals from peroxisomes may play a role in organelle, and thus, cell vitality. Below we summarize the current knowledge on preventing peroxisome-related physiological cell damage.

#### Peroxisomal ROS production

The many oxidative reactions in the peroxisome may lead to the formation of ROS. These include radical oxygen derivatives containing unpaired electrons, such as superoxide anion ( $O_2^{\bullet-}$ ), which is formed through one-electron reduction of  $O_2$  ( $O_2 + e^- \rightarrow O_2^{\bullet-}$ ) and  $H_2O_2$ . In peroxisomes, various oxidoreductases contribute to the generation of  $H_2O_2$  (Table 1; Schrader & Fahimi, 2006). In mammalian and plant peroxisomes,  $H_2O_2$  can also be formed by a dismutation reaction of  $O_2^{\bullet-}$  catalysed by superoxide dismutases via the hydroperoxyl radical ( $O_2^{\bullet-} + H^+ \rightarrow HO_2^{\bullet}$ ;  $2HO_2^{\bullet} \rightarrow H_2O_2 + O_2$ ). However, superoxide dismutases have, so far, not been observed in yeast peroxisomes.

Probably the most highly reactive and toxic form of ROS is the hydroxyl radical ( $\bullet OH$ ), which can be formed by metal ion (e.g. iron or copper)-catalysed decomposition of  $H_2O_2$  ( $H_2O_2 + O_2^{\bullet-} \rightarrow O_2 + OH^- + \bullet OH$ ) by the so-called Fenton reaction (Moldovan & Moldovan, 2004). Transition metal ions, such as iron and copper, are present in peroxisomes, where they are generally in complex with peroxisomal enzymes (e.g. as cofactor or in haeme). However, under certain conditions, these metal ions can be released from the enzymes and stimulate the formation of hydroxyl radicals.

A well-known toxic effect of ROS is the damage to biological membranes, which is initiated by lipid peroxidation. Common targets for peroxidation are unsaturated fatty acids present in membrane phospholipids. ROS can also cause carbonylation of proteins, which may result in loss of their function (Nguyen & Donaldson, 2005). Moreover, damage to DNA can cause mutations, which ultimately lead to nonfunctional proteins.

In living cells, damage of macromolecules by ROS is designated oxidative stress. It has been estimated that at least 1–2% of the oxygen used by an organism results in the generation of ROS, which is produced by mitochondria and peroxisomes (Bergamini *et al.*, 2004). Moreover, for rat liver, it has been estimated that peroxisomes produce about 35% of all  $H_2O_2$ , which accounts for about 20% of the total oxygen consumption in this tissue (Boveris *et al.*, 1972). Thus, ROS formation by peroxisomes may be an important factor in oxidative damage and ageing-related processes.

#### Role of peroxisomal antioxidant enzymes

Peroxisomes contain a number of ROS detoxification systems. All peroxisomal antioxidant enzymes can use  $H_2O_2$  as a substrate, thereby preventing the release of this compound from the organelle. However, each antioxidant enzyme has a different substrate specificity and affinity (e.g. the peroxiredoxin Pmp20 has a higher affinity for lipid hydroperoxides than for  $H_2O_2$ ; Horiguchi *et al.*, 2001b), which renders the overall antioxidant defence system more efficient and complete. This is important as any imbalance in the production and scavenging of ROS may result in damage to peroxisomal constituents or leakage of ROS from the organelles into the cytosol, thereby contributing to oxidative damage of other cellular components.

#### CAT

The antioxidant enzyme CAT uses  $H_2O_2$  as its main substrate ( $2H_2O_2 \rightarrow O_2 + 2H_2O$ ). The importance of CAT in yeast species is stressed by the observation that absence of the enzyme inhibits growth of yeast cells on methanol as a carbon and energy source (Hansen & Roggenkamp, 1989; Horiguchi *et al.*, 2001a). Furthermore, the presence of peroxisomal CAT in yeast results in a higher resistance to oxidative stress (cf. Cuéllar-Cruz *et al.*, 2008). The important role of peroxisomal CAT in ageing is best illustrated by the human disease hypocatalasemia. Hypocatalasemic patients have reduced CAT enzyme activities in their tissues, because they have one functional and one defective CAT allele (Góth *et al.*, 2004). These patients experience a premature onset of a number of age-related diseases (see Terlecky *et al.*, 2006 and the references therein). As might be expected, cells from hypocatalasemic patients accumulate

H<sub>2</sub>O<sub>2</sub> (Wood *et al.*, 2006). As a result, peroxisome function and matrix protein import can be severely compromised, defects that could be normalized upon artificial reintroduction of CAT protein (Sheikh *et al.*, 1998).

Analysis of the presence of CAT genes in the genomes of several budding yeasts (Table 2) indicates that all yeast species contain an ortholog of the peroxisomal *S. cerevisiae* Cta1 protein. Remarkably, only a few species have an ortholog of the cytosolic ScCtt1 protein. In *S. cerevisiae*, loss of peroxisomal Cta1 decreased the viability of the yeast cells 15-fold, relative to wild-type control cells, while loss of its cytosolic isoform Ctt1 had only a slight effect on the chronological life span of the cells (Petriv & Rachubinski, 2004). Similarly, in the nematode *Caenorhabditis elegans*, lack of peroxisomal CAT, but not of the cytosolic isoenzyme, accelerated ageing and resulted in a significant reduction in the mean life span (Petriv & Rachubinski, 2004).

These data clearly imply that the presence of active CAT enzyme results in increased viability. Nevertheless, this oxygen-scavenging pathway cannot prevent peroxisomal enzymes from ageing themselves. It has been shown in mammalian cells that ageing compromises peroxisomal protein import, with CAT itself being particularly affected (Legakis *et al.*, 2002). As a result, ageing cells produce increasing amounts of H<sub>2</sub>O<sub>2</sub>.

### Peroxisomal peroxiredoxins

Another family of proteins involved in detoxification of ROS consists of the peroxiredoxins, thioredoxin-dependent peroxidases that are conserved from yeast to humans (Immenschuh & Baumgart-Vogt, 2005). An analysis of the presence of peroxiredoxin genes in the genomes of budding yeasts (Table 2) indicates the existence of organelle-specific enzymes, which can be placed in four groups (PrxA to PrxD). Characteristic members of each group are the *S. cerevisiae* proteins Ahp1, Dot5, Prx1 and Tsa1. However, many paralogs exist. So far, none of the baker's yeast proteins has been localized to peroxisomes.

A well-studied member of the peroxiredoxin family is the PTS1 protein Pmp20, which belongs to group PrxA (ortholog of PrxA2; Table 2). This peroxisomal peroxiredoxin was first identified in the methylotrophic yeast *Candida boidinii* (see Horiguchi *et al.*, 2001b). *CbPmp20* is specifically induced during growth of cells on methanol and is exclusively present in peroxisomes. Deletion of *CbPMP20* resulted in a severe growth defect on methanol as a carbon and energy source. The PTS1 protein *CbPmp20* shows glutathione peroxidase activity, and is reactive against alkyl hydroperoxides and H<sub>2</sub>O<sub>2</sub>. Thus, it was speculated that the main function of the protein was to remove ROS damage generated at the peroxisomal membrane surface, for example in the form of lipid hydroperoxides (Horiguchi *et al.*,

2001b). We recently identified the Pmp20 ortholog in *H. polymorpha*, and demonstrated that in this yeast, Pmp20 is important for the viability of the cells (Bener Aksam *et al.*, 2008). Like in *C. boidinii*, *HpPmp20* has a PTS1 sequence, is localized to peroxisomes and the encoding gene is specifically induced on methanol. This suggests that the peroxiredoxin is only required during methylotrophic growth. Deletion of *HpPMP20* resulted in enhanced levels of ROS, lipid peroxidation and a severe growth defect on methanol. Moreover, the enhanced oxidative stress resulted in a specific leakage of proteins from peroxisomes, whose nature is unknown. Finally, these negative effects resulted in necrotic cell death.

Remarkably, next to *HpPmp20*, the putative *H. polymorpha* peroxiredoxin Hp47g996 also has a PTS1-related C-terminus, suggesting a peroxisomal localization. Both proteins cluster in the same group of peroxiredoxins (PrxA; Table 2; see Fig. 2 for an alignment of the C-termini of these proteins). Hp47g996 is the ortholog of *S. cerevisiae* Ahp1 (protein PrxA1a), which also contains a PTS1-related C-terminus. It has been shown that the subcellular location of *S. cerevisiae* Ahp1 depends on the growth conditions, i.e. being in the cytosol in glucose-grown cells, and in organelles – suggested to be mitochondria – in oleate-grown cells (Farcasanu *et al.*, 1999). We have recently found that its ortholog Hp47g996 is cytosolic when *H. polymorpha* cells are cultivated on methanol (B. de Vries *et al.*, unpublished data), implying that under methylotrophic conditions, *HpPmp20* is most likely the sole peroxisomal peroxiredoxin. Future research will have to determine whether Hp47g996, like ScAhp1, might have a growth condition-dependent subcellular location.

### Degradation of peroxisomal matrix proteins by peroxisomal proteases

The fate of nonfunctional or damaged peroxisomal proteins in the peroxisomal matrix is still largely unknown. Reports on the existence of peroxisomal proteases are rare and most are related to processing of PTS2 sequences from peroxisomal enzymes. Cleavage of the presequence of peroxisomal thiolase by insulin degrading enzyme (IDE) in mammalian peroxisomes has been reported by Authier *et al.* (1995). Recent data suggest that the main function of IDE in mammalian cells may be more related to hydrolysis of oxidized proteins (Morita *et al.*, 2000). In *S. cerevisiae*, the ortholog of this protein, designated Ste23, is not a peroxisomal protein. Ste23 is thought to be involved, together with its paralog Axl1, in the cytosolic N-terminal processing of pro- $\alpha$  mating factor into its mature form (Adames *et al.*, 1995). Interestingly, orthologs of IDE in some yeast species seem to possess a putative PTS1, but so

**Table 2.** Peroxisomal quality control proteins in budding yeasts

	Saccharomyces cerevisiae family				Candida albicans family				Yarrowia lipolytica		Hansenula polymorpha		Other	Loc
	Sc	Cg	Kl	Ag	Ca	Dh	Ps	Pg	Yl	Hp				
<b>Catalases</b>														
Cat1	CAA94095 (Cta1)	CAG61651	CAH00677	AAS54235	EAK99824	CAG89167	ABN64878	EDK37752	CAG78888	CAA39856	CtCat*	Per		
Cat2a	CAA97090 (Ctt1)	–	CAH00807	–	–	CAG85670	–	EDK41483	CAG80392	–	CbCat†	Cyt		
Cat2b	–	–	–	–	–	–	–	–	CAG80372	–	–			
<b>Lon-type proteases</b>														
mLon	CAA84841 (Pim1)	CAG60847	CAG99507	AAS53384	EAK98416	CAG89675	ABN68665	EDK36248	CAG78709	ABC69312	–	Mit		
pLon	–	–	CAH02008	AAS53253	EAK95632	CAG90071	ABN66580	EDK36584	CAG78606	ABB88892 (Pln)	–	Per		
<b>Peroxioredoxins‡</b>														
Prx1a	CAA61687 (Ahp1)	CAG62830	CAG98687	–	EAL01434	CAG90822	EAZ63560	EDK40480	CAG79980	Hp47g996	–	Unk		
Prx1b	–	–	–	–	–	CAG85297	–	–	–	–	–			
Prx2	–	–	–	–	–	–	–	–	–	ABY60796 (Pmp20)	CbPmp20§			
Prx3	–	–	CAH02911	AAS51766	EAK96847	CAG84391	EAZ63834	DNA	CAG82392	Hp7g47	–			
Prx4	–	–	–	–	EAL01973	CAG88329	ABN64488	EDK40778	–	–	–			
PrxB	CAA86239 (Dot5)	CAG58249	CAH00795	AAS51903	EAK98738	CAG87787	ABN64859	EDK38510	CAG79741	Hp8g460	–	Nuc		
PrxC1	CAA86627 (Tsa1)	CAG59604	CAH01996	AAS52992	EAK97074	CAG90466	ABN64593	EDK38683	CAG83166	Hp5g492	–	Cyt		
PrxC2	AAB64886 (Tsa2)	CAG61456	–	–	–	–	–	–	–	–	–			
PrxD1	CAA80784 (Prx1)	CAG57853	CAG99956	AAS54858	–	CAG86494	ABN65661	EDK39769	CAG77959	Hp15g933	–	Mit		
PrxD2	–	–	CAH02705	AAS51031	EAK97952	CAG89123	ABN67567	EDK41650	CAG84179	Hp15g633	–			

\* *Candida tropicalis* catalase (CtCat; accession number P07820).† *Candida boidinii* catalase (CbCat; accession number BAB69893).‡ Peroxioredoxins from budding yeast species were placed in four groups (PrxA to PrxD) based on their sequence similarity. Based on data obtained in *Saccharomyces cerevisiae* (Pedrajas et al., 2000; Izawa et al., 2004; Munhoz & Netto, 2004), the proteins of groups PrxB to PrxD probably have a nuclear (PrxB), cytosolic (PrxC) and mitochondrial (PrxD) localization in the cell. However, the subcellular localization of the proteins in the group PrxA is largely unknown.§ *Candida boidinii* Pmp20 (CbPmp20; accession number BAB43979).–, not present/identifiable; Cyt, cytosolic; Loc, subcellular location; Mit, mitochondrial; Nuc, nuclear; Per, peroxisomal; Unk, unknown. DNA: *Pichia guilliermondii* genomic sequence GenBank accession number AAFM01000038.1 nt 1790 to 544 (contains a frame-shift). Ag, *Ashbya gossypii*; Ca, *Candida albicans*; Cg, *Candida glabrata*; Dh, *Debaryomyces hansenii*; Hp, *Hansenula polymorpha*; Kl, *Kluyveromyces lactis*; Pg, *Pichia guilliermondii*; Ps, *Pichia stipitis*; Sc, *S. cerevisiae*; Yl, *Yarrowia lipolytica*.



ScAhp1	: YWGRWAVVENGIVTYAAKETNPGTDVTVSSVESVLAHL----	: 176	
CgCAG62830	: YWGRWTVVAKDGVVYAGNESNPASDVTSSVVEEAUKHL----	: 175	
KlCAG98687	: FWASRLVFAKDGKIVYQAVEENPATDVTSSVDNALEQLNKIK	: 171	
CaEAL01434	: LFLORYASIVV-NGEITYLETEDSLGFSESSAETILKRIHN--	: 176	
DhCAG90822	: IRTARYTSID-DGKVSFLESEGLGFSESSASTVLEHL----	: 173	PrxA1
DhCAG85297	: VRTSRYTAID-NGVIKYLESEDDGGFTSTNANDLLKKLD----	: 178	
PsEAZ63560	: VRTARYTAIV-DGEISFLENEDSLGFTESSAKNLLDRI----	: 177	
PgEDK40480	: IRTGRYATV-KDGKTEYLKAENGGSLLDTATAENLLGKL----	: 173	
YlCAG79980	: IRTARYATVD--DGKVTYNEQEPGKEVTVSGFDAY-AKL----	: 196	
Hp47g996	: YRTGRYATIAKDGVVTVYGVETG--KDVTVSSYEAVLEHL----	: 193	
<hr/>			
CbPmp20	: TRSRLALIVN-RSGIVEYAAIENGGEVDVSTAQKIAKL----	: 167	PrxA2
HpPmp20	: TRSRLALIVN-RSGIVEFVADEEGGVNVTASKLLAKL----	: 165	
<hr/>			
KlCAH02911	: DRSIRYAVIVQ-DGKVVKEFAEPDKIGVDYSSAENVLKEK----	: 181	
AgAAS51766	: DRSVRYALVVE-DGKVVRFVPEPKTGKVSAAENVLKEK----	: 197	
CaEAK96847	: ERSKRYALVE-DGKVVKSFVEPDNTSDVSAAQVLEEA----	: 184	
DhCAG84391	: ERSKRYALVE-DGKVTETFEVDNISVDVSDAEKVLSCV----	: 185	PrxA3
PsEAZ63834	: ERSRRYALVE-DGTVTKTFVEPDNTSDVSDSANVLEEA----	: 166	
PgAAM01000038	: HRSKRYALVD-DGKVVKTFFVEPDNVSDVSAAEKVLQV----	: 181	
YlCAG82392	: KRSARYAVTM-DGKVVVEFVEPDKTGDDVSKAENVLKSIG----	: 162	
Hp7g47	: VRSKRYCLAVD-DNKVVAEFVEPQNTFVDVTAADKLFKQLPGL	: 187	
<hr/>			
CaEAL01973	: IRTGRYATVD-DSKTKDVVYLGVEVEKGVQSSGIDAVL-AKL----	: 185	
DhCAG88329	: IRTARYATVVEDKTRKISYLGKEVERGVKFSGIDAVLQAKL----	: 185	PrxA4
PsABN64488	: IRTSRYATVDSKDKTVKYFGVEVERGVKFSGLDVLGAKL----	: 194	
PgEDK40778	: IRTARYATVFS-KDRRVKYVGKEVDRSVRYSGVEAVV-AKL----	: 188	

**Fig. 2.** Alignment of C-terminal sequences of the peroxiredoxins from group PrxA. For abbreviations and GenBank accession numbers, see Table 2. Sequences were aligned using the CLUSTAL\_X program. Gaps were introduced to maximize the similarity. Residues that are similar in all proteins are shaded black. Residues that are similar in at least 19 sequences are shaded dark grey, while residues that are similar in at least 14 sequences are shaded light grey. Putative PTS1 sequences are marked in red. Based on sequence conservation, the peroxiredoxins of the PrxA type can be divided into four distinct subgroups PrxA1 to PrxA4. Components of subgroups PrxA2 (methylotrophic Pmp20 orthologs) and PrxA4 (proteins from *Candida albicans*-related species) might all represent peroxisomal proteins based on the presence of the highly conserved -AKL-COOH sequence. Proteins in subgroup PrxA3 lack a PTS and therefore are presumably not peroxisome-borne. In certain members of subgroup PrxA1, a PTS1-related C-terminus is present, but this is not fully conserved.

far no information as to the subcellular location of these proteins is available.

Stewart *et al.* (2002) described a protease activity in peroxisomes of the methylotrophic yeast *C. boidinii*, but the corresponding gene has so far not been identified. Also in plant peroxisomes endoprotease activities were identified (Distefano *et al.*, 1999). More recently, the peroxisomal protease Tysnd1 has been characterized in mouse peroxisomes. This enzyme processes PTS1- and PTS2-containing enzymes involved in  $\beta$ -oxidation of fatty acids (Kurochkin *et al.*, 2007). However, yeast orthologs do not seem to exist.

In none of the above-mentioned studies was the proteolytic process related to cellular housekeeping. However, recently, a peroxisomal Lon protease was identified in rat (Kikuchi *et al.*, 2004) – as well as a putative ortholog in *H. polymorpha* (Aksam *et al.*, 2007) – which most likely does have such a function. Lon protease was initially characterized in *Escherichia coli* (Charette *et al.*, 1981). Subsequently, this protease has been identified in most organisms and its structure has been shown to be remarkably well conserved throughout evolution. ATP-dependent Lon proteases are multidomain enzymes that contain an ATPase domain belonging to the AAA(+) superfamily and a proteolytic domain with a serine–lysine catalytic dyad. In human cells, the mitochondrial Lon protease (mLon) was initially discovered as the protease that degrades oxidatively modified

aconitase (a TCA cycle enzyme known to be susceptible to oxidative inactivation; Bota & Davies, 2002). mLon is located in the mitochondrial matrix, where free radical generation from the electron transport chain can be extremely high. So far, mLon is the best-characterized protease known to degrade damaged organellar matrix proteins. Several data suggest that mLon levels decrease with age. Davies and colleagues showed that downregulation of mLon levels results in loss of mitochondrial function, reduced mitochondrial biogenesis and eventual cell death (see Ngo & Davies, 2007 and the references therein). In addition, mitochondrial morphological pathologies similar to those seen in ageing cells were observed, suggesting that reduced mLon levels may contribute to ageing.

All yeast species – with the exception of *S. cerevisiae* (and relatives) and *Candida glabrata* – contain two Lon protease isoenzymes, one of which is potentially mitochondrial and the other is probably peroxisomal (Table 2; Aksam *et al.*, 2007). The peroxisomal Lon protease (pLon) of *H. polymorpha* is a PTS1 protein that appears to play a role in the degradation of unfolded peroxisomal matrix proteins. Moreover, the absence of pLon in this yeast resulted in an increase in intracellular ROS levels, increased levels of carbonylated (oxidized) proteins and a decreased viability of the cells (Aksam *et al.*, 2007). Most probably, pLon is involved in housekeeping of peroxisomes, preventing/restoring damage caused by ROS generated in the organelles.

Recently, two other functions have been proposed for the mammalian ortholog. Yokota *et al.* (2008) suggested that pLon may function in degrading  $\beta$ -oxidation enzymes upon recovery of cells from drug-induced peroxisome proliferation. Another study indicated that the protein might contribute to sorting and processing of PTS1 proteins (Omi *et al.*, 2008).

### Organelle quality control: removal of entire peroxisomes by pexophagy

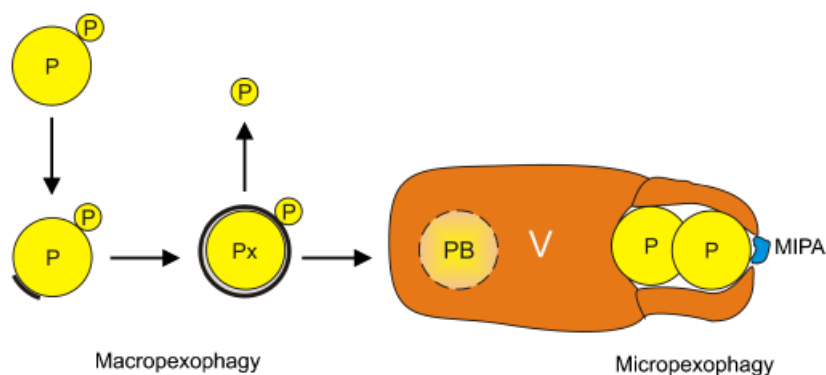
In addition to quality control mechanisms that prevent/repair oxidative damage to peroxisomal proteins, entire peroxisomes are also subject to quality control and can be degraded by processes related to autophagy.

The term autophagy refers to a multitude of processes that result in the uptake of cytoplasmic components into vacuoles (in fungal and plant cells) or lysosomes (the equivalent to vacuoles in mammalian cells), organelles that contain a large assortment of hydrolases (see Klionsky *et al.*, 2007). Autophagy is thought to be important for house-keeping to prevent accumulation of damaged cell constituents. In addition, autophagy contributes to cell survival, when cells are placed under nutrient limitation conditions and recycle part of their cytoplasmic components. Furthermore, autophagy allows cellular remodelling because entire organelles can be degraded when these become redundant.

Two main autophagy mechanisms are known: macroautophagy (and related processes) and microautophagy (and related processes). These pathways differ in the mechanism by which they deliver substrates to the vacuole lumen, the types of substrates transported and their activity/regulation

(Klionsky *et al.*, 2007). Both macro- and microautophagy are responsible for the degradation of long-lived proteins and whole organelles, and can be induced under stress conditions, such as starvation. During macroautophagy, a portion of the cytoplasm, often including entire organelles, is surrounded by a double membrane, thus forming an autophagosome. Fusion of the vacuole with autophagosomes exposes the contents of the autophagosomes to the vacuolar hydrolases, resulting in their degradation. Microautophagy involves the direct engulfment of portions of the cytoplasm by the vacuolar membrane, without prior formation of an autophagosome. This occurs either by direct invagination or through formation of finger-like protrusions that sequester cytoplasmic components. Once the engulfment is complete, the membrane and the sequestered material are degraded. In addition to these nonselective macro- and microautophagy processes, many morphologically related, but highly selective processes are known that utilize the same machinery to allow specific cargo to enter the vacuole, for example the Cvt pathway in *S. cerevisiae* (and *P. pastoris*) that utilizes the autophagy machinery to provide the vacuole with the enzyme aminopeptidase I (Scott *et al.*, 1996; Farré *et al.*, 2007). Also, organelles such as peroxisomes use macro- or microautophagy-related pathways to selectively enter the vacuole for turnover (processes that have collectively been designated pexophagy; Fig. 3; for a review, see Sakai *et al.*, 2006).

Selective degradation of peroxisomes by macro- and micropexophagy has been studied mainly in methylotrophic yeasts such as *H. polymorpha* and *P. pastoris*. When grown on methanol as the sole carbon source, methylotrophic yeast species contain several large peroxisomes that contain the key



**Fig. 3.** Selective degradation of peroxisomes in yeast by macro- and micropexophagy. When peroxisomes are no longer required for growth (e.g. during a shift to a medium containing a carbon source that does not require peroxisomes), or have become damaged, the organelles can be specifically degraded. Left: During macropexophagy, a single mature peroxisome (P) becomes sequestered by additional membranes. After sequestration is complete, the outer layer of the now formed pexophagosome (Px) fuses with the vacuolar membrane. Upon entry into the vacuole (V), the resulting pexophagic body (PB) will be degraded by vacuolar hydrolases. Usually, one or a few import-competent peroxisome(s) remain(s), which can function as the progenitor(s) of new organelles when required. Right: During micropexophagy, the vacuole starts to invaginate and septate, thereby engulfing a cluster of peroxisomes. Before complete sequestration of the organelles, a novel membrane structure designated micropexophagy-specific membrane apparatus (MIPA) is formed, which mediates fusion between the tips of the invaginating vacuole. Subsequently, lysis of the interior membrane releases the peroxisomes into the lumen of the vacuole, where they are degraded.

enzymes for methanol utilization (AO, dihydroxyacetone synthase and CAT). Pexophagy occurs when methanol-grown cells are shifted to a new carbon source, whose metabolism does not involve these peroxisomal enzymes (e.g. glucose or ethanol), but the actual degradation mechanism is species and inducer dependent. During macropexophagy, individual peroxisomes are selectively sequestered to form pexophagosomes, which subsequently fuse with the vacuole, resulting in degradation of the entire organelle (Fig. 3; Kiel *et al.*, 2003). When peroxisomes are degraded by micropexophagy, the organelles are directly engulfed by vacuoles without prior sequestration (Fig. 3; Farré & Subramani, 2004). Despite these significant morphological differences, many of the molecular players (so-called Atg proteins; Klionsky *et al.*, 2003) are required for both macro- and micropexophagy.

Only recently, data have been presented that indicate that autophagic degradation of peroxisomes in yeast also takes place as part of cellular housekeeping (Aksam *et al.*, 2007). It was observed that during chemostat cultivation of wild-type *H. polymorpha* cells, entire peroxisomes were constitutively degraded by autophagy during normal vegetative growth. Probably, this autophagic degradation enables the cells to rejuvenate the peroxisome population. Indeed, under the same conditions, *atg1* mutants had enhanced peroxisome numbers as well as increased intracellular levels of ROS relative to wild-type controls. This enhanced ROS generation occurred concurrent with an uneven distribution of peroxisomal CAT activity in the mutant cells. These observations are strikingly similar to senescent mammalian cells, which have increased numbers of morphologically altered peroxisomes with a decreased matrix protein content (especially PTS1 proteins; Legakis *et al.*, 2002). Autophagy is most likely the major mechanism of peroxisomal protein housekeeping, because the half-life of peroxisomal proteins was not affected by the absence of peroxisomal Lon protease (Aksam *et al.*, 2007).

## Concluding remarks

Like cytosolic proteins, luminal and membrane proteins from organelles such as peroxisomes are under continuous quality control. During their cytosolic residence, they face the same threats as cytosolic proteins. However, in certain cases, specific proteins are in place that aid in maintaining the enzymes in an import-competent state and/or prevent them from aggregation (e.g. Pex19 for PMPs and Pyc1 for AO). Also, inside the peroxisome, the proteins face specific threats. The oxidases in peroxisomes can produce significant quantities of ROS (e.g. AO in methanol-grown cells) that damage proteins and lipids. Mechanisms are in place to remove these dangerous oxygen derivatives or repair oxidized proteins/lipids (e.g. peroxiredoxins). If this is not

possible, peroxisome-specific proteases (e.g. pLon) may degrade the damaged proteins. Finally, to prevent the occurrence of dysfunctional peroxisomes, pexophagy can remove entire organelles, so that only the optimally functioning ones remain to support growth and survival of the cell.

A growing body of evidence suggests that peroxisomes are important organelles in maintaining cell viability, a role that has long been neglected. One of the reasons might be that the mitochondrion was the major topic in studies related to cell viability, cell death and ageing. However, depending on the organism, growth conditions and tissue, peroxisomes may also be significant ROS producers and, as a consequence, important for these processes.

It is important to realize that all pathways in the cell may be somehow connected to each other. Mitochondria and peroxisomes may functionally interact via ROS signaling. This possible interaction between these organelles should be investigated more carefully to determine their individual role in ageing and cell death processes such as apoptosis and necrosis. It has been shown that ROS can act as signalling molecules in various cellular pathways including programmed cell death. The potential function of peroxisomes in inducing these pathways is an important topic for future research and very relevant for medicine.

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